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On-line trace enrichment in hyphenated liquid chromatography–nuclear magnetic resonance spectroscopy

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Abstract

The potential of on-line trace enrichment combined with column liquid chromatography–nuclear magnetic resonance detection (LC–NMR) is demonstrated for the identification of hop bitter acids in hop extracts. By performing on-line solid-phase extraction on 1–5 ml of hop extract (which contains the six major hop bitter acids) the detection/analysis time in the stopped-flow detection mode could be reduced 4-fold, from 60 min to 15 min per individual peak. The applicability of combined trace enrichment and separation using a single short (12.5 mm×4.6 mm I.D.) high-pressure-packed column combined on-line with NMR was also studied. Sample enrichment of 5 ml of a solution spiked with three drugs at 5 μg/ml was sufficient for the acquisition of high-quality spectra both in the stopped-flow and even in the continuous-flow detection mode. The examples demonstrate that analyte detectability can be significantly improved by performing on-line sample enrichment prior to LC–NMR analysis.

Keywords: Nuclear magnetic resonance spectroscopy; Trace enrichment; Solid-phase extraction, on-line; Hop extract; Naproxen; Fenprofen; Ibuprofen; Hop bitter acids

1. Introduction

Hyphenation of powerful separation and detection techniques plays an important role in the development of novel analytical methods. In the past few years, a new alternative has been added to the long list of hyphenated techniques, column liquid chromatography–nuclear magnetic resonance spectroscopy (LC–NMR) [1]. Advances made with regard to flow-cell and NMR interface designs and the progress made in solvent-suppression techniques by applying in pulse sequences [2] to allow the use of protonated solvent systems have played a major role here. Although NMR is a very powerful spectroscopic technique and provides detailed structural and stereochemical information, its rather poor sensitivity compared with, e.g., mass spectrometry (MS) is a major drawback. In addition, it is difficult to elucidate the structure of unknown organic compounds in a complex matrix because of overlapping ^{1}H NMR signals which cause overcrowding of signals in a small chemical shift region of the NMR spectrum. LC separation prior to detection helps to eliminate the latter problem. However, the fact remains that NMR is an inherently insensitive detection tech-

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nique. In practice, this means that analyte concentration is necessary.

One way to obtain reliable analytical data at the trace level is to perform trace enrichment prior to (chromatographic) separation. Nowadays, on-line solid-phase extraction (SPE) is often used to concentrate the compounds of interest from aqueous samples. The on-line coupling of SPE–LC with diode array detection (DAD)–UV [3,4], electrochemical [5] and mass spectrometric [6,7] detection is routinely used to identify and/or quantify organic microcontaminants in surface and tap water, and the full automation of such systems has been reported. In view of the above consideration, it lies at hand to try to combine SPE and LC–NMR in one on-line set-up. In order to explore the potential of this approach, two completely different examples were studied: (i) stopped-flow and (ii) continuous-flow detection/characterization. On-line SPE of hop bitter substances in hop extract with LC–NMR can significantly increase analyte detectability and will consequently decrease total analysis time (in both the stopped-flow and continuous-flow detection mode).

Recently, a novel on-line SPE–LC approach was developed in which both SPE and analytical separation are performed on a single short (10–20 mm in length) high-pressure packed LC column, the so-called single-short-column (SSC) approach [8,9]. Using this approach in combination with different detection systems including MS, the total analysis time could significantly be reduced without too much loss in chromatographic efficiency.

In the present communication, the potential of combining on-line SPE and LC–NMR is demonstrated. It is shown that a considerable gain in analyte detectability can be achieved in the stopped-flow as well as the continuous-flow detection mode compared with conventional NMR. Furthermore, the applicability of the SSC–NMR offers the possibility of rapid identification of compounds.

2. Experimental

2.1. Chemicals

Acetonitrile, methanol (both HPLC-grade) and phosphoric acid (85% analytical-reagent grade) were purchased from J.T. Baker (Deventer, The Netherlands). Water was in-laboratory distilled in an all-quartz system and deuterium oxide (99.8%) was obtained from Deutero (Herresbach, Germany). Supercritical CO_2 hop extracts were gifts of Hopstabil (Munich, Germany) and Heineken (Zoeterwoude, The Netherlands). The drugs were purchased from Sigma (St. Louis, MO, USA).

2.2. Sample preparation

10% (m/m) solutions of the supercritical CO_2 hop extract were prepared by weighing 1 g and mixing with 9 g methanol for 10 min. The white precipitate was removed by filtration (0.2 μm pores). This solution was diluted 500 times in water–methanol (80:20, v/v) giving a 0.02% solution which was used for injections.

Stock standards solutions of the drugs (naproxen, Ca-fenoprofen, ibuprofen) were prepared by dissolving 10 mg of each compound in 10 ml of HPLC-grade methanol. Standard mixtures were prepared by dilution of the stock solutions with quartz-distilled water to 5 μg/ml. These mixtures were used for injections.

2.3. Instrumentation and conditions for LC–UV

2.3.1. Analysis of hop extract

LC–UV was performed using an HP1100 system (Hewlett-Packard, Waldbronn, Germany) equipped with a vacuum degasser, a binary solvent delivery system, an autosampler (equipped with a 900-μl syringe), a thermostatted column compartment, and a UV–Vis DAD system. The LC system was controlled by a personal computer (Hewlett-Packard Vectra XM p5/120) using ChemStation software (Hewlett-Packard). SPE was performed on a 10 mm×2 mm I.D. cartridge packed with 15–25 μm PLRP-S (Spark Holland, Emmen, The Netherlands). The cartridge was built into a laboratory-made holder. For sample enrichment the HP1100 autosampler equipped with the 900-μl syringe was used in the multidraw mode. This autosampler was controlled by a HP1100 Control Module. The LC separation was carried out on a Microsphere column of 100 mm×4.6 mm I.D. packed with 3 μm C_{18} bonded silica (Chrompack, Bergen op Zoom, The

Netherlands). The eluents were (A) water–0.05% phosphoric acid (85%) and (B) acetonitrile–0.05% phosphoric acid (85%); gradient elution of 60% B to 80% B in 20 min was performed at a flow-rate of 1 ml/min. The detector wavelength was 330 nm.

2.3.2. Analysis of drugs

For the drugs, the same LC system was used as described in Section 2.3.1 for the hop extracts. Both sample enrichment and chromatographic separation were performed on a 12.5 mm×4.6 mm I.D. Zorbax-SB column packed with 5 μm C_{18} bonded silica (Rockland Technologies, Nuenen, The Netherlands). The trace-enrichment procedure was similar to SSC approaches performed with earlier LC set-ups [7–9]. In short, the SSC is conditioned with methanol in order to wet the packing material and then activated with aqueous eluent, i.e., initial gradient composition, to obtain suitable conditions for analyte sorption. Subsequently, a known volume is loaded on the column. For the 12.5 mm column, prior to applying 1–10 ml samples, the column was conditioned with 5 ml methanol and 5 ml initial eluent (both steps: 5 min at flow-rate of 1 ml/min). The gradient was of 5% B to 95% B in 10 min at a flow-rate of 1 ml/min and the detector was set at a wavelength of 215 nm.

2.4. Instrumentation and conditions for LC–UV–NMR

A Merck–Hitachi (Darmstadt, Germany) L-6200A intelligent pump, a Merck–Hitachi L-4000A UV detector and a Bruker (Rheinstetten, Germany) Peak Sampling Unit BPSU-12 were used. The system was controlled by Bruker LC-Chromstar software and the Bruker LC244M interface. The outlet of the BPSU was connected by a PEEK capillary to a 200 μl continuous flow probe with a radio frequency coil arranged for inverse $^{13}C/^{1}H$ spectroscopy. The spectrometer was a Bruker ARX 400 (9.4 Tesla) equipped with Bruker UXNMR software. The LC gradient/eluent conditions were the same as used in the LC–UV approach, but for detection with NMR the water in eluent A was replaced by deuterium oxide. In case of detection in continuous-flow mode of the drugs, the eluent composition was (isocratic) 30% B during the complete analysis with a flow-rate of 0.3 ml/min.

The spectra were recorded at 297 K. The number of transients which were accumulated for the spectra depended on the concentration and/or the volume of the samples that were enriched.

The spectra were processed using WIN-NMR. Manual baseline correction was applied to all spectra.

3. Results and discussion

3.1. SPE–LC–NMR for the analysis of hop bitter acids in hop extracts

For the analyses of the hop bitter acids, a supercritical CO_2 hop extract sample was used from which the fatty acids and the waxes were previously separated by precipitation with methanol. The LC conditions were chosen in accordance with those previously used for these compounds [10] and then optimized for the present system. Fig. 1 shows the chromatogram of an SPE–LC–UV separation. In this case, 5 ml of 0.02% (m/m) of hop extract in water–methanol (80:20, v/v) were preconcentrated on a PLRP-S cartridge which was situated in front of the analytical column. This preliminary experiment was performed to obtain information about the response/sensitivity for detection in NMR.

For on-line SPE–LC–UV–NMR the water in the eluent was replaced by deuterium oxide. To be able to compare our results with those published earlier without SPE being involved, the ^{1}H NMR spectra of the hop bitter acids were recorded in the stopped-flow mode. The UV detector was used to determine when a compound (top of the peak) is in the flow probe of the NMR. To observe the effect of sample enrichment, 1 ml and 5 ml of a 0.02% (m/m) hop extract were loaded on the SPE pre-column. In case of the 1-ml sample, the ^{1}H NMR spectra were recorded with 1 *k* (=1024) accumulated scans for each peak. The time needed to record 1 k scans is about 60 min. If 5 ml of sample were loaded (also see below), the number of scans could be reduced to 256, and only 15 min were required per peak. As an example, the ^{1}H NMR spectrum for the 5-ml sample of cohumulon is shown in Fig. 2. The spectra of all six hop bitter acids were closely similar to those obtained in the earlier LC–NMR analyses [10]; they

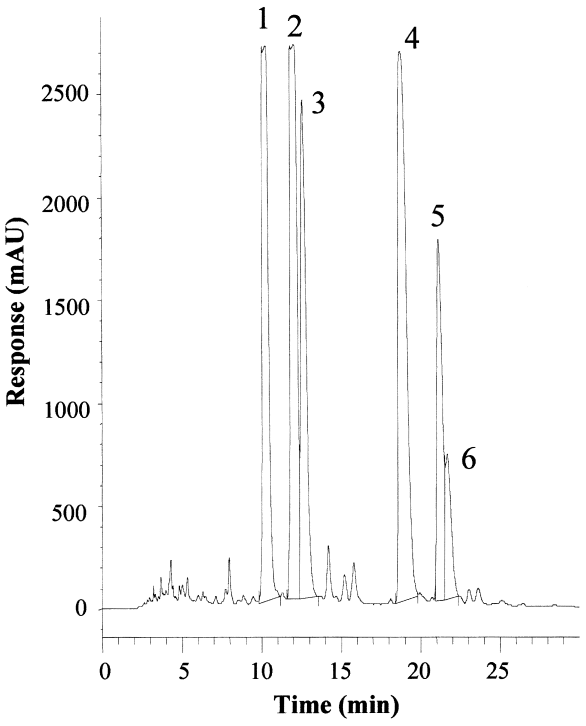

Fig. 1. Stopped-flow SPE–LC–UV chromatogram of 5 ml 0.02% hop extract showing the separation of six major hop bitter acids. Sampling flow-rate, 1 ml/min. Conditioning with 5 ml of methanol and 5 ml of initial eluent; clean-up with 5 ml of initial eluent. Linear gradient from A–B (40:60, v/v) to (20:80, v/v) in 20 min at a flow-rate of 1 ml/min. Column: Microsphere column of 100 mm×4.6 mm I.D. packed with 3 μm C_{18} bonded silica. Peak assignment: 1=cohumulon, 2=humulon, 3=adhumulon, 4=colupulon, 5=lupulon, 6=adlupulon. For other conditions, see Section 2.3.1.

are in agreement with their known structures and, therefore, are not discussed here. However, it has to be added that because of the less than optimal LC separation of four of the hop bitter acids, the spectrum of adhumulon also showed signals due to humulon; this was even more so for lupulon and adlupulon. This implies that it will be rather difficult to obtain spectra of all six hop bitter acids in the continuous-flow mode under the present gradient conditions, also because changes in solvent composition during elution will cause chemical shifts in the spectra which will severely interfere with the compound of interest. Further studies should therefore be focused on the possibility of obtaining better-quality spectra by increasing the performance of the LC part of the set-up.

When using the HP1100 autosampler to inject the large sample volumes, it takes about 3 min to load 1 ml of sample on a SPE cartridge, and 12 min to load 5 ml of sample. As a consequence, performing on-line sample enrichment for 9 min more to load 5 ml of sample instead of 1 ml, results in a decrease of recording time of 45 min for each peak, viz. 60 min versus 15 min. For the analysis of all six hop bitter acids a decrease in total analysis time of ca. 4 h 30 min can be achieved.

3.2. SSC–NMR for the analysis of drugs

The on-line combination of SSC and NMR was studied for the analysis of some model compounds, naproxen, fenoprofen and ibuprofen. First, the LC eluent composition was optimized for the chromatographic separation of the three drugs on the short column. After preconcentration of 5 ml of solution containing with 5 μg/ml of each of the drugs on top of the 12.5 mm short column, the actual chromatographic separation was performed using a gradient from 5% B to 95% B in 10 min at 1 ml/min. These injections at higher concentration levels were performed to investigate whether the concentrations would be high enough to obtain high-quality NMR spectra.

In the on-line SSC–UV–NMR experiments, the water in the eluent was replaced by deuterium oxide. First, the ^{1}H NMR spectra of all three drugs were recorded in the stopped-flow mode. To this end, 5 ml of a 5 μg/ml mixture were loaded onto the 12.5 mm short column and on-column separated with the (optimized) LC eluent and on-line transferred to the NMR system. Each ^{1}H NMR spectrum was recorded from 256 accumulated spectra, giving a spectrum of ibuprofen as shown in Fig. 3. Allowing for different solvents and spectrometer frequencies, these data are in agreement with ^{1}H NMR data of ibuprofen reported earlier [11,12]. Furthermore, all data are in agreement with the known structures of the analyte(s) and conventionally obtained spectra. The signals in the aromatic region are important for structure elucidation of these different drugs. Whereas ibuprofen has a *para*-substituted phenyl ring, fenoprofen has a *meta*-substituted one and naproxen

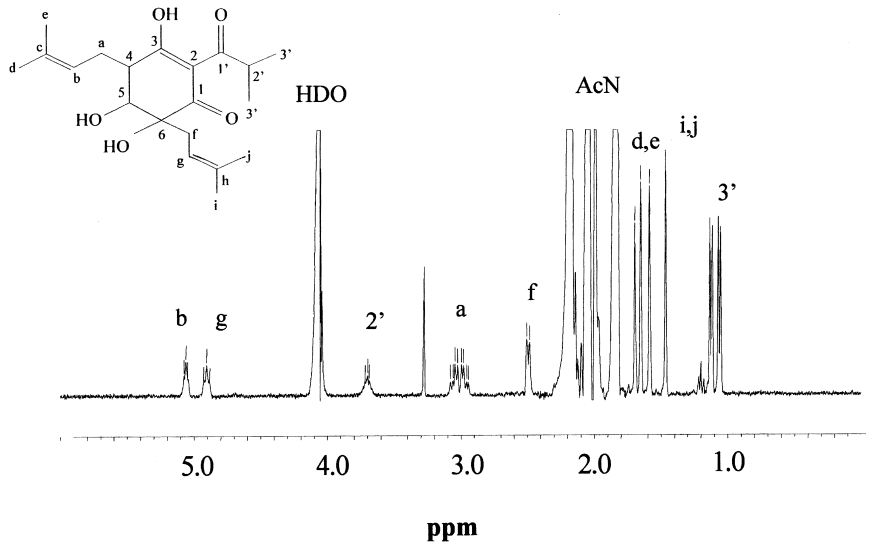

Fig. 2. SPE–LC–NMR cohumulon stopped-flow mode spectrum recorded after trace enrichment of 5 ml of 0.02% hop extract. Two hundred and fifty six scans were accumulated. Peak assignment is in accordance with the numbers given in the structure of cohumulon. For LC conditions, see Fig. 1. For other conditions, see Sections 2.4 and 3.2.

is a substituted naphthelene. Although the three drugs are completely separated in the SSC–UV chromatogram, the separation was not quite sufficient to obtain pure spectra in NMR without any interference from the other analytes. The stopped-flow mode combined with the long capillary (ca. 2 m×0.17 mm I.D.) between the UV detector and the NMR system causes some more longitudinal diffusion resulting in extra band broadening and, therefore, a poorer separation in the NMR system than in the UV detector. Under the present conditions, the NMR spectrum of naproxen was pure, but the ^{1}H NMR spectrum of fenoprofen showed some interference from naproxen and that of ibuprofen from fenoprofen (see insert of Fig. 3). The total analysis time for trace enrichment, separation and detection using stopped-flow recording for all three drugs was about 70 min.

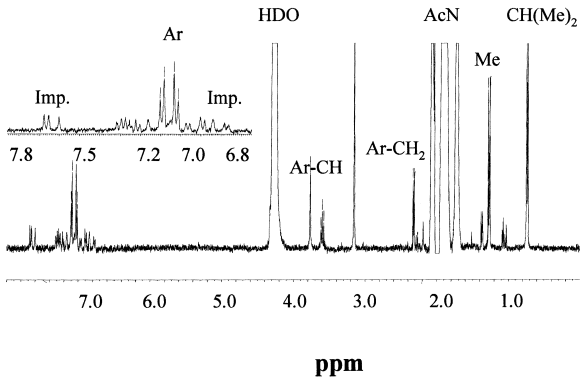

Fig. 3. SSC–NMR ibuprofen spectrum obtained after trace enrichment of 5 ml of a solution of three drugs, recorded in the stopped-flow mode. Two hundred and fifty six scans were accumulated. Conditioning with 5 ml of methanol and 5 ml of initial eluent (both steps: 5 min at flow-rate of 1 ml/min). Linear gradient from 5% B to 95% B in 10 min at flow-rate of 1 ml/min. Column: 5 μm Zorbax-SB C_{18}, 12.5 mm×4.6 mm I.D. Peak explanations: Ar, aromatic signals; Me, methyl signals, Imp., impurities of fenoprofen and naproxen. For structure of ibuprofen, see Fig. 6.

To obtain information on analyte detectability with the present set-up, on-line trace enrichment and separation were also performed with 5 ml of a 1 μg/ml solution. In this case 1 *k* of scans had to be accumulated to obtain NMR spectra having the same quality as before, and the total analysis time increased to ca. 3 h 30 min, which we consider the upper limit for an analytical run.

Another mode to record the NMR spectra of the drugs is the continuous-flow mode. Continuous-flow detection offers the possibility of acquiring structural

information in a much more time-efficient way. NMR characterization/detection after LC gradient elution is still difficult because of reasons mentioned before; however, when isocratic elution is used, this drawback is absent. Of course, the amount of analyte necessary to obtain a reliable spectrum will be higher than in the stopped-flow mode. In the continuous-flow mode SSC–NMR experiments 10 ml, rather than the earlier 1–5 ml, of the 5 μg/ml solution were therefore concentrated and subjected to analysis. The actual chromatographic separation was performed with an isocratic eluent containing 30% B at a flow-rate of 0.3 ml/min. The NMR recorded 64 spectra/h at 32 accumulated scans/spectrum. The total analysis time of enrichment, separation and recording was about 60 min. Figs. 4 and 5 show the SSC–UV chromatogram and the corresponding two-dimensional (2D) plot, respectively. Especially the position of signals of the aromatic protons is im-

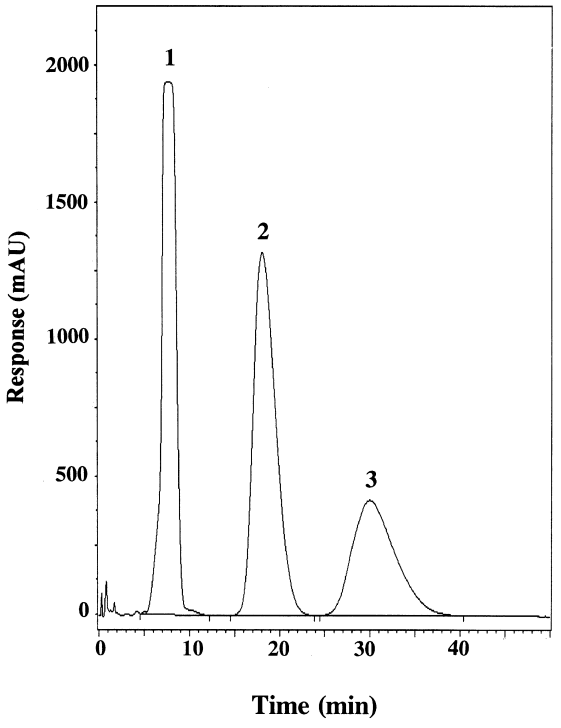

Fig. 4. Continuous-flow SSC–UV chromatogram obtained after trace enrichment of a 10-ml sample spiked with 5 μg/ml of each naproxen, fenoprofen and ibuprofen on a 12.5 mm×4.6 mm I.D. column. Sampling flow-rate, 1 ml/min; elution flow-rate, 0.3 ml/min. Conditioning with 5 ml of methanol and 5 ml of A–B (95:5, v/v). Isocratic elution with A–B (70:30, v/v). Peak assignment: 1=naproxen, 2=fenoprofen and 3=ibuprofen. For other conditions, see Section 2.3.2.

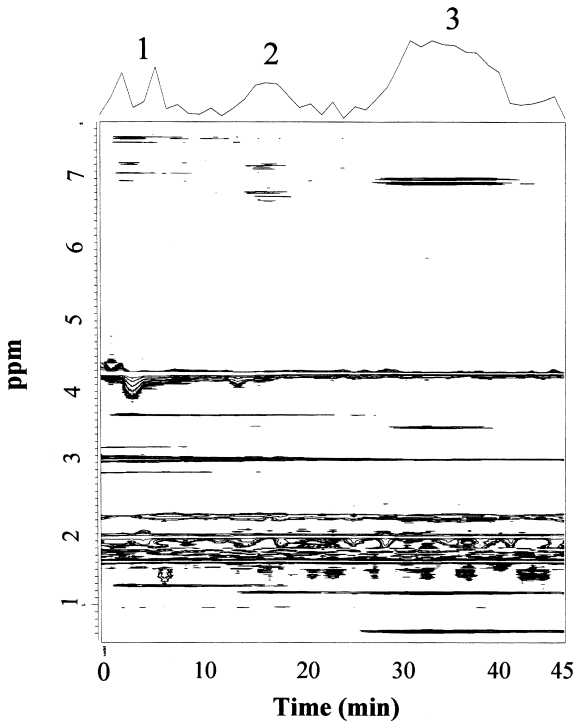

Fig. 5. 2D plot of continuous-flow SSC–NMR, 64 rows/h; 32 accumulated scans/spectrum. Peak assignment: 1=naproxen, 2=fenoprofen and 3=ibuprofen.

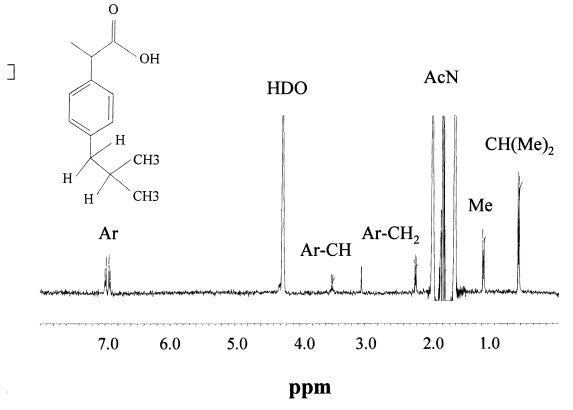

Fig. 6. NMR spectrum of ibuprofen obtained by extracting one row from the 2D plot of Fig. 5. For peak explanation, see Fig. 3 and given structure of ibuprofen. For other conditions, see Sections 2.4 and 3.2.

portant in the identification of the three substances. Different rows can be extracted from this on-line NMR spectrum to receive a conventional 1D NMR spectrum. Fig. 6 shows the spectrum of ibuprofen; to this end, one row was extracted from the 2D plot of Fig. 5. This ^{1}H NMR obtained in the continuous-flow mode shows the pure spectrum of ibuprofen without impurities of the other drugs. The total analysis time is somewhat shorter despite the larger sample volume that has to be loaded. Actually the time gain of the SSC–NMR approach can be increased if a faster sample loading set-up such as the Prospekt is used instead of the present, rather slow modified HP1100 autosampler.

4. Conclusions

On-line trace enrichment in hyphenated LC–NMR was demonstrated for the separation and detection of hop bitter acids in hop extracts. The resolution of the ^{1}H spectra in the stopped-flow mode is similar to that of spectra recorded after conventional LC, and identification of the compounds of interest can easily be achieved. By performing sample enrichment a significant gain in analyte detectability can be achieved. Increasing the sample volumes 5-fold, viz. from 1 to 5 ml, causes the total analysis time for an individual compound to decrease by a factor of 4. For the six hop bitter acids of interest this amounts to a dramatic gain of 4 h and 30 min per run.

If a limited number of analytes has to be studied, the so-called SSC approach can also be used. The on-line SSC–NMR combination offers the possibility for extremely rapid trace enrichment, separation and NMR detection/identification – in the present instance, of three drugs. Results obtained in both the stopped-flow and continuous-flow mode showed the potential for the rapid collection of detailed structural information. These results are considered extremely promising. Further studies are planned to refine the present strategy. Future experiments will be focused on thoroughly studying the various effects of sample volume versus analyte concentrations and spectral acquisition time in on-line SPE–LC–NMR and SSC–NMR.

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